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FORM PTO-1390 (REV. 3-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 447.001
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/806368
INTERNATIONAL APPLICATION NO. IB99/01621	INTERNATIONAL FILING DATE October 4, 1999	PRIORITY DATE CLAIMED October 9, 1998	
TITLE OF INVENTION BONE MORPHOGENETIC PROTEIN ANTAGONIST BASED ON THE MATURE PROTEIN			
APPLICANT(S) FOR DO/EO/US KATSUURA et al			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none">1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none">a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau) in Englishb. <input type="checkbox"/> has been transmitted by the International Bureau.c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none">a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).b. <input type="checkbox"/> have been transmitted by the International Bureau.c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.d. <input type="checkbox"/> have not been made and will not be made.8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). Unexecuted10. <input type="checkbox"/> A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
Items 11. to 16. below concern document(s) or information included:			
<ol style="list-style-type: none">11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.14. <input type="checkbox"/> A substitute specification.15. <input type="checkbox"/> A change of power of attorney and/or address letter.16. <input checked="" type="checkbox"/> Other items or information: International Preliminary Examination Report; Drawings (2 sheets); Sequence Listing (paper copy) part of application			

USPTO RECEIVED 28 MAR 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/806368		INTERNATIONAL APPLICATION NO. PCT/IB99/01621		ATTORNEY'S DOCKET NUMBER 447.001	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1070.00				\$ 500.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$930.00					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$790.00					
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$720.00					
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$98.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 500.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	- 20 =		x \$22.00	\$	
Independent claims	- 3 =		x \$82.00	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$ 500.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				+	
SUBTOTAL =				\$ 500.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$ 500.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
TOTAL FEES ENCLOSED =				\$ 500.00	
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a. <input checked="" type="checkbox"/> A check in the amount of \$ 500.00 to cover the above fees is enclosed.					
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Bierman, Muserlian and Lucas 600 Third Avenue New York, NY 10016					
					
20311					
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SIGNATURE					
Charles A. Muserlian					
NAME					
19,683					
REGISTRATION NUMBER					

Our Ref.: 447.001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:
PCT/IB99/01621	: PCT Date: April 10, 1999
Mieko KATSUURA et al	:
Serial No.:	:
Filed: Concurrently Herewith	:
For: BONE...MATURE PROTEIN	:
	600 Third Avenue
	New York, NY 10016

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Page 1, before line 1, insert

--This application is a 371 of PCT/IB99/01621 filed
October 4, 1999.--

IN THE CLAIMS:

Claim 8 (amended) The mature protein according to claim 1,
wherein said mature human MP52 is a dimer protein.

Claim 11 (amended) The mature protein according to claim 9,
wherein said mature human BMP-2, mature human BMP-4, or mature
human BMP-7 is a dimer protein.

application, to remove multiple dependency from the claims and to
add method of use claims.

Respectfully submitted,
BIERMAN, MUSERLIAN AND LUCAS



Charles A. Muserlian, #19,683
Attorney for Applicant(s)
Tel. # (212) 661-8000

CAM:sd

Enclosures: Marked up Version of Claims
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What is claimed is:

1. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.
2. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an oxidization reaction.
3. The mature protein according to claim 2 in which four methionine residues are oxidized and having the amino acid sequence of SEQ ID N° 5.
4. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an alkylation reaction.
5. The mature protein according to claim 4 wherein the alkylation reaction is S-carboxymethylation in which at least one methionine residue is S-carboxymethylated and having the amino acid sequence of SEQ ID N° 6.
6. The mature protein according to claim 1, wherein the chemical modification for said tryptophane residue is an allylsulphenylation reaction.
7. The mature protein according to claim 6 in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID N° 7.
8. The mature protein according to ^{claim 1} ~~claims 1 to 7~~, wherein said mature human MP52 is a dimer protein.
9. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3) or mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.

10. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by replacing at least one amino acid residue of three hydrophobic amino acid residues, among said hydrophobic amino acid residues relating
5 to a receptor binding site in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4), which are located in positions corresponding to those of methionine residues located in 30th, 71st, and 74th positions of the amino acid
10 sequence of mature human MP52 (SEQ ID N° 1) with a hydrophilic amino acid residue or a polar amino acid residue.
11. The mature protein according to claim 9 ~~or 10~~, wherein said mature human BMP-2, mature human BMP-4, or mature human BMP-7 is a dimer protein.
- 15 12. An agent for therapy and/or prevention of ectopic ossification, containing a mature protein according to ~~any one of claims 1 to 11~~ ^{claim 1} as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.
- 20 13. An agent for therapy and/or prevention of metabolic diseases with calcification, containing a mature protein according to ~~any one of claims 1 to 11~~ ^{claim 1} as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

21pts

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MATURE PROTEIN HAVING ANTAGONISTIC ACTIVITY AGAINST BONE
MORPHOGENETIC PROTEIN

BACKGROUND OF THE INVENTION

5 (1) Field of the Invention

This invention relates to a mature bone morphogenetic protein of which some hydrophobic amino acid residues are exchanged to a hydrophilic or a polar amino acid residue by chemical modification or genetic engineering technology. The mature proteins of this invention show an antagonistic activity against bone morphogenetic proteins, and are useful for medicinal agents to suppress symptoms of ectopic osteogenesis and ectopic calcification or metabolic bone diseases with calcification such as neurotic osteosis, ectopic ossification caused by stress of operation, traumatic myositis ossificans, ossification by defect of oxygen supply, osteogenic tumor, ossification of the posterior longitudinal ligament, and arterial sclerosis.

(2) Description of the Related Art

20 A bone morphogenetic protein (hereinafter called BMP) is a protein having a bone morphogenetic activity in decalcified bone tissue. Although the isolation of BMP had been worked on energetically since the 1970s, it was quite difficult to isolate as a single protein. Gene cloning of BMP as expected was performed by Wozney in 1989 by molecular biological technology, using the amino acid sequences derived from unknown peptides which were separated by treating the fraction having bone morphogenetic activities with an enzyme. The gene was immediately introduced to the animal cultured cells, and the activity of the protein expressed was measured in vivo, and BMP activity in the protein was practically proved (Wang, E. A. et al., (1990) Proc. Natl. Acad. Sci. USA, vol. 87, p. 2220-2224). Continuing cloning a protein with bone morphogenetic activities utilizing homology, several numbers of the proteins with bone morphogenetic activities in a similar structure have been isolated so far. Those proteins all belong to TGF (transforming growth factor)- β superfamily and are proved to have the activity to

It seems that suppression of BMP existence may be one of the major treatments for these diseases. Another treatment, such as administration of BMP antagonists, also seems
35 effective. BMP receptors, neutralized antibodies against BMP and the BMP peptides corresponding to the binding site, a BMP with chemically modified specific amino acid residue are thought to have BMP antagonist-like activity.

Many studies have been so far carried out concerning a relationship between the structure and the activity of BMPs and it is speculated that some sites of mature BMPs relate to the receptor binding. It is known that a peptide synthesized
5 based on these studies works as an antagonist against BMPs (JP patent application, Hei 7 ('95) - 200175).

SUMMARY OF THE INVENTION

Other than the synthetic peptides, many kinds of methods are desired to exist for therapy of various diseases of bones
10 and cartilages such as hyperplasia. The purpose of this invention is to provide a new BMP antagonist protein as an effective therapeutic agent for above bone-related diseases in which a specific amino acid residue is chemically modified or replaced by genetic engineering technology.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a comparison of ALPase inducing activities by methionine alkylated mature human MP52s and that of unmodified mature human MP52 in MC3T3-E1 cells, which were separated by the difference in the retention time on the
20 reverse phase HPLC. In the figure, the solid line with solid circles represents unmodified mature human MP52, the solid line with solid squares represents one-methionine alkylated mature human MP52, the broken line with solid triangles represents two-methionine alkylated mature human MP52, the
25 solid line with open circles represents three-methionine alkylated mature human MP52, the dotted line with open triangles represents four-methionine alkylated mature human MP52, respectively. The solid circles shown in of the Y-axis represents ALPase activity in cells without treated by any
30 reagent.

Fig. 2 shows the antagonistic activity of methionine oxidized mature human MP52 and tryptophane allylsulphenylated mature human MP52 in different cell lines. (A) represents their antagonistic activities against mature rh-BMP-2 in
35 C3H10T $\frac{1}{2}$ cells and (B) shows their antagonistic activities against mature human MP52 in MC3T3-E1 cells, respectively. In both figures, the solid line with solid circles represents tryptophane allylsulphenylated mature human MP52 and the

dotted line with open circles represents methionine oxidized mature human MP52, respectively. In (A), the solid square shows ALPase activity induced by 300 ng/ml of mature rh-BMP-2 alone in C3H10T $\frac{1}{2}$ cell. In (B) the solid square shows ALPase activity induced by 600 ng/ml of unmodified mature human MP52 alone in MC3T3-E1 cells. Open squares represent the activity in the experiments without any reagent.

DESCRIPTION OF THE PREFERRED EMBODIMENT

10 The present invention relates to a mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic
15 residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue by genetic engineering technology.

When at least one residue of said methionine is converted, chemical modification for said methionine residue
20 is an oxidization reaction or an alkylation reaction.

When the chemical modification of said methionine residue is an oxidization reaction, among the preferred mature proteins of the invention is that in which four methionine residues are oxidized and having the amino acid
25 sequence of SEQ ID N° 5.

When the chemical modification of said methionine residue is an alkylation reaction, among the preferred mature proteins of the invention is that in which at least one methionine residue is S-carboxymethylated and having the
30 amino acid sequence of SEQ ID N° 6.

When at least one residue of said tryptophane is converted, chemical modification for said tryptophane residue is an allylsulphenylation reaction.

Among the preferred mature proteins of the invention is
35 that in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID N° 7.

The present invention relates to mature proteins of the invention as defined above having an antagonist activity

Chemical modification of this invention means that the side chains of methionine or tryptophane are modified in a degree enough to make the protein inactive with keeping a binding ability to the receptor. For the chemical
 5 modification, a conventional method in studies on protein chemistry is applied. The method is described in "Protein Chemistry IV," pp 6-118, 1977, Jap. Soc. Biochem. Methionine is a hydrophobic amino acid and can be modified into a hydrophilic residue by chemical modification. Chemical
 10 modifications in increasing hydrophilicity are exemplified by oxidization of methionine to make methionine sulfoxide or methionine sulfon using an oxidant and by alkylation to make S-carboxymethylmethionine using α -halogenoacetic acid. The oxidization of methionine are performed by hydrogen peroxide,
 15 performic acid, periodic acid, N-chlorosuccinimide, and N-bromosuccinimide. Besides, for α -halogenoacetic acid, monoiodoacetic acid and monoiodoacetamide can be used.

Chemical modification to indole ring of tryptophane gives a subtle change of environment and structure nearby it.
 20 Suitable examples are allylsulphenylation of the second position of indole nucleus by p- or o-nitrophenylsulphenylchloride, 2-nitro-4-carboxyphenylsulphenylchloride, 2,4-dinitrophenylsulphenylchloride, or 2-hydroxy-5-nitrobenzylation of the 3rd position of indole nucleus by 2-
 25 hydroxy-5-nitrobenzylbromide, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonylbromide.

The present invention in particular relates to a mature protein of which one to 4 methionine residues or 1 to 2 tryptophane residues are chemically modified in mature human
 30 MP52 amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing.

The protein shown in SEQ ID N° 1 of the Sequence Listing can be produced by the method described in International Patent Application WO 96/33215. Since 3 out of 4 methionine
 35 residues exist in the receptor binding site of mature human MP52 produced by said method, and all 2 tryptophane residues exist there, and whose amino acids are all hydrophobic, it is assumed that these hydrophobic amino acids play an important

role in bone morphogenetic activity.

Mature human MP52 of the present invention includes not only the mature protein with the amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing but also Ala or Arg-Ala
5 binding to the N-terminus of the amino acid sequence of said mature protein as disclosed respectively in international patent application WO 95/04819 and WO 97/06254.

Comparing the amino acid sequences of the mature proteins of other BMPs such as human BMP-2 (SEQ ID N° 2),
10 human BMP-4 (SEQ ID N° 3), human BMP-7 (SEQ ID N° 4) and so on with that of mature human MP52 protein, it is found that in those bone morphogenetic proteins the amino acids corresponding to the positions of methionine residues of mature human MP52 are all methionine residues or hydrophobic
15 amino acids.

In concrete, the receptor binding site of mature human BMP-2 is estimated to be the peptides with the amino acid sequence from 16th Arg to 34th Ala and from 56th Asn to 73th Lys shown in SEQ ID N° 2 of the Sequence Listing (Science
20 242, 1528-1534, 1988). The amino acids of mature human BMP-2, which corresponds to methionines' position of the mature human MP52 (30th, 71st and 74th), are replaced with 26th Val, 67th Val and 70th Val.

The receptor binding site of mature human BMP-4 is
25 estimated to be the peptides with the amino acid sequence from 18th Arg to 36th Ala and from 58th Asn to 75th Ser shown in SEQ ID N° 3 of the Sequence Listing (DNA Seq. 5 (5), 272-275, 1995). The amino acids of mature human BMP-4, which corresponds to methionines' position of mature human MP52
30 (30th, 71st and 74th), are replaced with 28th Val, 69th Val and 72rd Val.

The receptor binding site of mature human BMP-7 is estimated to be the peptides with the amino acid sequence from 40th Lys to 58th Ala and from 80th Asn to 97th Glu shown
35 in SEQ ID N° 4 of the Sequence Listing (Proc. Natl. Acad. Sci. U.S.A. 93(2), 878-883, 1996). The amino acids of mature human BMP-7, which corresponds to methionines' position of mature human MP52 (30th, 71st and 74th), are replaced with

50th Leu, 91st Val and 94th Ile.

It is difficult to carry out a chemical modification selectively on the hydrophobic amino acids of which positions correspond to those of methionine of mature human MP52.

- 5 However, it is possible to replace hydrophobic amino acids with hydrophilic or polar amino acids by genetic engineering technology.

Since the tryptophane residues are conserved in all BMPs described above, it is possible to replace hydrophobic amino
10 acids with hydrophilic or polar amino acids by chemical modification method or genetic engineering technology. The tryptophane residues also play an important role in making a change in a three-dimensional structure in chemical modification and genetic engineering technology.

- 15 Thus, the present invention relates to mature human MP52, in particular, which is obtained by converting one to 4 methionine residues or one to 2 tryptophane residues existing in the amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing to hydrophilic residues or polar amino acid
20 residues by recombinant engineering technology. In the present invention hydrophilic amino acids and polar amino acids are aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on. In the present invention, when methionine residues or tryptophane residues
25 are converted to other hydrophilic or polar amino acids, the protein of the present invention can be obtained by transforming a recombinant DNA into *E. coli* which are made by replacing the codons corresponding to methionines at 30th, 71st, 74th and 111th positions or the codons corresponding to
30 tryptophanes at 32nd and 35th positions shown in SEQ ID N° 1 replaced with the codons of aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on or by making.

- Further, the present invention relates to any mature
35 proteins having the amino acid sequence shown in SEQ ID Nos 2 to 4, of which one to two tryptophane residues are subjected to chemical modification.

The present invention furthermore relates to any mature

proteins having the amino acid sequence shown in SEQ ID Nos 2 to 4, in which hydrophobic amino acids consisting of the receptor binding site, 1 to 3 hydrophobic amino acids corresponding to the positions of 30th, 71st and 74th methionine residues or 1 to 2 tryptophane residues are replaced with hydrophilic amino acids or polar amino acids. In the present invention, hydrophobic amino acids are methionine, valine, leucine, isoleucine or tryptophane.

The present invention relates to the mature protein having the amino acid sequence shown in SEQ ID N° 5 of the Sequence Listing, in which 4 methionine residues of the methionine residues are oxidized. In detail, for the oxidization of methionine residue, hydrogen peroxide is added at a final concentration of 0.014% to 2 mg/ml of the mature protein and the reaction is carried out for more than 15 hours at room temperature and then the mature protein of which methionine residues are in methionine sulfoxide can be obtained.

The present invention relates to the mature protein having the amino acid sequence shown in SEQ ID N° 6 of the Sequence Listing, in which 1 to 4 methionine residues of the methionine residues are alkylated. In detail, moniodoacetic acid is added at a concentration of higher than methionine residues by 50 to 100 at the molar ratio to 2 mg/ml of the mature protein and the reaction is carried out for more than 15 hours at room temperature and then the mature protein of which methionine residues are S-carboxymethylated can be obtained.

The present invention relates to the mature protein having the amino acid sequence shown in SEQ ID N° 7 of the Sequence Listing, in which 2 tryptophane residues of the tryptophane residues are allylsulphenylated. In detail, 20 equivalents of p-nitrophenylsulphenyl chloride in 100% acetic acid is added to 2 mg/ml of the mature protein and the reaction is carried out for 1 hour at room temperature and then the mature protein of which tryptophane residues are allylsulphenylated can be obtained.

The present invention relates to the mature protein

having an amino acid sequence shown in SEQ ID N° 2 of the Sequence Listing, in which 1 to 2 tryptophane residues at 28th and 31st are allylsulphenylated.

The present invention relates to the mature protein
5 having an amino acid sequence shown in SEQ ID N° 2 of the Sequence Listing, in which one or all of 26th, 67th and 70th valine residues or one or both of 28th and 31st tryptophane residues are replaced with aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on.

10 The present invention relates to the mature protein having an amino acid sequence shown in SEQ ID N° 3 of the Sequence Listing, in which one or both of 30st and 33th tryptophane residues are allylsulphenylated.

The present invention relates to the mature protein
15 having an amino acid sequence shown in SEQ ID N° 3 of the Sequence Listing, in which one or all of 28th, 69th and 72rd valine residues or one or both of 30st and 33th tryptophane residues are replaced with aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on.

20 The present invention relates to the mature protein having an amino acid sequence shown in SEQ ID N° 4 of the Sequence Listing, in which one or both of 52nd and 55th tryptophane residues are allylsulphenylated.

The present invention relates to the mature protein
25 having an amino acid sequence shown in SEQ ID N° 4 of the Sequence Listing, in which one or all of 50th leucine, 91st valine, 94th isoleucine, 131th methionine or one or both of 52nd and 55th tryptophane residues are replaced with aspartic acid, glutamic acid, lysine, arginine, histidine, serine,
30 threonine and so on.

The BMP antagonist-like activity of the protein modified chemically or replaced with other amino acids in the present invention can be proved by measuring alkaline phosphatase (ALPase) activity as a marker of biological activity by
35 adding in a culture medium of a murine carvarial clonal cell line (MC3T3-E1 cell) having osteoblastic property which Kodama et al. established (Kodama, H. et al. (1981) Jpn. J. Oral Biol., vol. 23, p.899). ALPase is often used as a

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dissolved in water. And after putting the mixture into vials or ampoules, they are freeze-dried and then sealed to be an injection preparation.

Although the adult clinical administration dosage for a day may vary from and also depend on administrating methods, ages, weigh, conditions of patients and so on, it is usually 0.01 - 5 mg of the protein.

This invention is described in detail by examples written below. However, this invention is not restricted to these examples.

EXAMPLES

Example 1 Preparation of mature human MP52 of which methionine residue is oxidized

(1) Oxidization of methionine residue of mature human MP52 by hydrogen peroxide (conversion to methionine sulfoxide)

Hydrogen peroxide was added to mature human MP52 (2 mg/ml concentration) dissolved in 2 mM EDTA - 10 mM hydrochloric acid at the final concentration of 0.014% and the solution was reacted at room temperature for more than 15 hours.

(2) Separation of methionine oxidized mature human MP52

Mature human MP52 having the amino acid sequence shown in SEQ ID N° 1 (hereafter, unmodified mature human MP52) and methionine oxidized mature human MP52 having the amino acid sequence of SEQ ID N° 5 were separated by using reverse phase HPLC on the basis of a difference between retention times of both proteins. Oxidization increased a hydrophilic property of the proteins and retention time methionine oxidized mature human MP52 on the reverse phase HPLC becomes faster than that of unmodified mature human MP52. The conditions of separation are as follows. For column, Nucleosil 5-C18-300 column (4.6 mm I.D. X 150 mm, GL Science Corp.) was used at the flow rate of 1.3 ml per minute at 45°C; absorbance at 214 nm and 280 nm were measured to detect peaks. For solvent, water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of proteins were performed with a linear gradient of solution B from 25% to 45% for 80 minutes using an HPLC pump, HP1050

20 First, lyophilized unmodified mature human MP52 and methionine oxidized mature human MP52 were dissolved in 8 M urea - 0.2 M ammonium bicarbonate - 2 mM EDTA (pH 8.5) at the final concentration of 1 mg/ml, and 50-fold molar excess of dithiothreitol (DTT) to the cysteine residue was added and
25 reacted at 50°C for 30 minutes. To this solution, 250-fold molar excess of monoiodoacetamide to the cysteine residue was added and reacted for 30 minutes with shading to yield S-carboxymethylated unmodified mature human MP52 and S-carboxymethylated methionine oxidized mature human MP52.
30 Digestion of these proteins was performed by adding trypsin. After four-fold dilution of this solution with water to make the final urea concentration of 2 M, at the weight ratio of 1/50 to the proteins at 37°C for 18 hours, the trypsin digestion was applied to the reverse phase HPLC column to
35 separate all fragments. The conditions of separation are as follows. Nucleosil 5-C18-300 column (4.6 mm I.D. X 150 mm, GL Science Corp.) was used for separation at the flow rate of 1.3 ml per minute at 45°C; absorbance at 214 nm was measured

to detect peaks. For solvent, water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of peptides was performed by a linear gradient of solution B from 0% to 45% for 90 minutes after
5 keeping 0% for initial five minutes, using HPLC pump, HP1050 (Hewlett Packard).

Subsequently, amino acid composition analysis was carried out to determine the positions of the digested peptide on the primary structure of reduced alkylated
10 unmodified mature human MP52 and reduced alkylated methionine oxidized mature human MP52. The operation of the amino acid composition analysis was mainly based on Zoku Seikagaku Zikken Kouza (Tokyo Kagaku Doujin), Vol. 2, Protein Chemistry (I), Section 4. Brief description is given below.

15 Hydrolysis was performed in a vapor of 6 N HCl containing 0.1% phenol at 110°C for 21 hours by using PICO. TAG. WORK STATION (Waters). Following this step, the amino acid composition analysis was carried out by PTC method by using Amino acid standard H (Pierce) as a standard amino acid.
20 PTC-amino acids were separated by reverse phase HPLC using HPLC pump (model 510; Waters), a Wakopak WS-PTC (4.0 mm I.D. X 200 mm; Wako Pure Chemicals), and solvents of PTC amino acids eluent A and PTC amino acids eluent B (both Wako Pure Chemicals).

25 The HPLC retention times of the identified trypsin peptides on the primary structures of unmodified and oxidized mature MP52 were compared each other. The trypsin fragments of mature human MP52 containing methionine residues were three kinds corresponding to: position from 29th to 56th (29-
30 56; containing 30th methionine) shown in SEQ ID N° 1, position from 57th to 88th (57-88; containing 71st, and 74th methionines) in SEQ ID N° 1, and position from 107th to 119th (107-119; including 111th methionine) in SEQ ID N° 1. The elution time of respective fragments derived from the reduced
35 alkylated unmodified mature human MP52 were around 84 min, 62 min, and 36 min in order. In comparison, the fragments derived from the reduced alkylated methionine oxidized mature human MP52 (SEQ ID N° 5) were around 80 min, 48 min, and 31

min in order showing the earlier elution than that of the fragments derived from the reduced alkylated unmodified mature human MP52.

On the other hand, there was no difference for other fragments which do not contain methionine. As a result, the specific reaction of oxidization to the methionine residues was determined.

Example 2 Preparation of mature human MP52 of which methionine residue was S-carboxymethylated

(1) Alkylation of methionine residue of mature human MP52 by moniodoacetic acid

Though the highest reactivity of alkylation using moniodoacetic acid is observed in SH residues of cysteine residue, the alkylation reaction almost selectively occurs in methionine residue in an acidic condition, because all the cysteine residues of unmodified mature human MP52 form disulfide bonds as described before. Therefore, the operation was conducted as follows. Moniodoacetic acid (molar ratio; 50-100 times higher than moles of methionine residue) was added to unmodified mature human MP52 with a concentration of 2 mg/ml dissolved in 10 mM hydrochloric acid and incubated at room temperature for 3-18 hours.

(2) Separation of mature human MP52 of which methionine residue was alkylated

Unmodified mature human MP52 and methionine alkylated mature human MP52 having the amino acid sequence of SEQ ID N° 6 were separated on the basis of a difference in retention times between both proteins by using reverse phase HPLC. The conditions of separation (a column employed, a flow rate, wavelengths for detection, and a pump for HPLC) were the same as those of Example 1 (2). For solvent, water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of the proteins were performed by a linear gradient of solution B from 30% to 45% for 60 minutes after five minutes of an initial condition.

Under this condition, unmodified mature human MP52 is eluted as one peak around 38 minutes and, in contrast to this, alkylated mature human MP52 showed four peaks (elution

Similar to Example 1 (3), cysteine residues were blocked by alkylation before trypsin digestion after reduction of disulfide bonds by using dithiothreitol. Since S-carboxymethylation was performed using monoiodoacetic acid for the alkylation of methionine; alkylation of cysteine was performed by using 4-vinyl pyridine as an alkylation reagent for monoiodoacetic acid, which was not similar to Example 1. Alkylation reaction takes place almost selectively in reduced

cysteine at pH 8.5, and hardly in methionine. Thus, the reason of employing different alkylation is to detect side reaction in methionine.

First, lyophilized unmodified mature human MP52 and
 5 methionine alkylated mature human MP52 were dissolved in a solution of 6 M guanidine - HCl - 0.4 M Tris-HCl buffer (pH 8.5) at the final concentration of 1 mg/ml, and reduced with 50-fold excess molar of dithiothreitol (DTT) to the cysteine residues at 50°C for 30 minutes. S-pyridyl-ethylation was
 10 performed by adding 250-fold excess molar of 4-vinyl pyridine to the cysteine residues and incubated for 30 minutes with shading. The solution was applied on a desalting column (PD-10; Pharmacia) equilibrated with a 6 M guanidine - HCl - 0.4 M Tris-HCl buffer (pH 8.5) to remove excessive reagent
 15 and followed by reverse phase HPLC using a reverse phase column (Cosmasil 10C18-300, 4.6 mm I.D. X 100 mm, Nakalai Tesque Inc.). A fraction having both absorption at 280 nm derived from tryptophane and 254 nm derived from pyridine by UV detector was collected.

20 Following this step, the trypsin digestion was performed by the same method as that of Example 1 (3) to separate digested fragments.

Finally, the position of respective fragments in the primary structure of mature MP52 was determined by the amino
 25 acid composition analysis as same as that of Example 1 (3).

Using the reverse phase HPLC, a comparison of elution times of respective trypsin fragments of unmodified mature human MP52 and methionine alkylated mature human MP52 of which position in the primary sequences was determined by the
 30 amino acid composition analysis was performed. Trypsin fragments of mature human MP52 containing a methionine residue were three kinds corresponding to: the amino acid position from 29th to 56th (29-56; containing 30th methionine) shown in SEQ ID N° 1, the amino acid position
 35 from 57th to 88th (57-88; containing 71st and 74th methionines) in SEQ ID N° 1, and the amino acid position from 107th to 119th (107-119; containing 111th methionine) in SEQ ID N° 1. The elution time of respective fragments derived

from unmodified mature human MP52 were around 77 min, 58 min, and 36 min in order. In comparison, fragments derived from methionine alkylated mature human MP52 (SEQ ID N° 6) were around 74 min, 42 min, and 30 min in order, showing the earlier elution than that of the fragments of unmodified mature human MP52.

On the other hand, the elution time of other fragments without containing methionine was the same between unmodified and methionine alkylated mature human MP52.

In the amino acid composition analysis, only the number of methionine residues derived from the methionine alkylated mature human MP52 was different from the theoretical values. The N-terminal sequence of these fragments were analyzed by using a sequencer (model 476A; Applied Biosystems) and it is confirmed that the alkylation occurred to methionine residues specifically.

Example 3 Preparation of mature human MP52 of which tryptophane was allylsulphenylated

(1) Allylsulphenylation of tryptophane residues of mature human MP52 by p-nitrophenylchloride

Twenty-fold molar ratio of p-nitrophenylchloride, which dissolved in 100% acetic acid, was added to the unmodified mature human MP52 (2 mg/ml dissolved in 50% acetic acid) and incubated at room temperature for 1 hour.

(2) Separation of allylsulphenylated mature human MP52

Unmodified mature human MP52 and allylsulphenylated mature human MP52 having the amino acid sequence of SEQ ID N° 7 were separated on the basis of a difference in the retention time of column on the reverse phase HPLC. The elution time of allylsulphenylated mature human MP52 delays in comparison with unmodified mature human MP52 on the reverse phase HPLC, which is due to increase in hydrophobic property by allyl sulphenylation. The conditions of separation are as follows; a column employed, a flow rate, and a pump for HPLC were the same as those of Example 1 (2), and column temperature and wavelengths for detection were 40°C, and 214 nm and 365 nm, respectively. For solvent, water containing 0.05% TFA as solution A and acetonitrile

containing 0.05% TFA as solution B were used for elution. The elutions of proteins were performed by a linear gradient of solution B from 25% to 60% for 35 minutes after maintaining for five minutes with 25% of solution B. Under
5 the conditions, unmodified mature human MP52 was eluted around 22 min, and in comparison, tryptophane allylsulphenylated mature human MP52 was eluted around 26 min. The difference of elution times between these proteins allows easy separation of them.

10 (3) Determination of allylsulphenylation of tryptophane

After reduction of disulfide bond by the same method described in Example 1, unmodified mature human MP52 of which cysteine residues were alkylated (S-carboxymethylated) and tryptophane allylsulphenylated mature human MP52 were
15 digested by trypsin, and fragments of the digest were separated by C18 reverse phase column. Amino acid positions of the fragments were determined by the amino acid composition analysis.

The trypsin fragment of mature human MP52 containing
20 tryptophane residues was that corresponding to the position from 29th to 56th positions (29-56; containing 32nd and 35th positions of tryptophane) alone shown in SEQ ID N° 1. Only the elution time (around 95 min) of a fragment containing tryptophane residue, which is derived from allylsulphenylated
25 mature human MP52 (SEQ ID N° 7) delayed in comparison with the elution time (around 84 min) of a fragment derived from the unmodified mature human MP52. On the other hand, the elution time of other fragments without containing tryptophane showed no change. N-terminal sequence analysis
30 showed difference only in tryptophane residue between unmodified and modified mature human MP52.

Example 4 Test of inhibition of bone morphogenetic activity

BMP-2 antagonistic activity of mature protein of this invention was tested by adding the protein to culture media
35 of MC3T3-E1 cells which are a murine carvarial clonal cell line established by Dr. Kodama et al. and have osteoblast-like properties, or C3H10T $\frac{1}{2}$ cells which are a mesenchymal and multipotent cell line to differentiate to osteoblast,

Fig. 2 shows the antagonistic activity of methionine oxidized mature human MP52 obtained in example 1 and tryptophan allylsulphenylated mature human MP52 obtained in

As shown in Fig. 2(A), 300 ng/ml of mature rh-BMP-2 promoted ALPase activity in C3H10T 1/2 cell about 40 times higher than the control group. The modified mature human MP52 of this invention inhibited the ALPase activity from 1 equivalent- to 20 equivalent-molar in a dose-dependent manner. Moreover, as shown in Fig. 2(B), 600 ng/ml of the unmodified mature human MP52 promoted ALPase activity in MC3T3-E1 cells about 3 times higher than the control group. The amino acid modified mature human MP52 of this invention inhibited the ALPase activity from 1 equivalent- to 10 equivalent-molar in a dose-dependent manner.

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23

Sequence Listing Free Text

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- <210> 2
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- <210> 3
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111th Met are modified to Met sulfoxide.
- <210> 6
20 <223> Mature MP52 protein. Note : 30th and/or 71st
and/or 74th and/or 111th Met are modified to
s-carboxymethyl Met.
- <210> 7
25 <223> Mature MP52 protein. Note : 32nd and 35th Trp are
modified to allylsulphenyl Trp.

What is claimed is:

1. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least
5 one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.
- 10 2. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an oxidization reaction.
3. The mature protein according to claim 2 in which four methionine residues are oxidized and having the amino acid
15 sequence of SEQ ID N° 5.
4. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an alkylation reaction.
5. The mature protein according to claim 4 wherein the
20 alkylation reaction is S-carboxymethylation in which at least one methionine residue is S-carboxymethylated and having the amino acid sequence of SEQ ID N° 6.
6. The mature protein according to claim 1, wherein the chemical modification for said tryptophane residue is an
25 allylsulphenylation reaction.
7. The mature protein according to claim 6 in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID N° 7.
8. The mature protein according to claims 1 to 7, wherein
30 said mature human MP52 is a dimer protein.
9. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature
35 human BMP-4 (SEQ ID N° 3) or mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.

10. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by replacing at least one amino acid residue of three hydrophobic amino acid residues, among said hydrophobic amino acid residues relating to a receptor binding site in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4), which are located in positions corresponding to those of methionine residues located in 30th, 71st, and 74th positions of the amino acid sequence of mature human MP52 (SEQ ID N° 1) with a hydrophilic amino acid residue or a polar amino acid residue.

11. The mature protein according to claim 9 or 10, wherein said mature human BMP-2, mature human BMP-4, or mature human BMP-7 is a dimer protein.

12. An agent for therapy and/or prevention of ectopic ossification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

13. An agent for therapy and/or prevention of metabolic diseases with calcification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/IB99/01621 (22) International Filing Date: 4 October 1999 (04.10.99) (30) Priority Data: 10/288103 9 October 1998 (09.10.98) JP (71) Applicant (for all designated States except US): HOECHST MARION ROUSSEL [FR/FR]; 1, terrasse Bellini, F-92800 Puteaux (FR). (72) Inventors; and (75) Inventors/Applicants (for US only): KATSUURA, Mieko [JP/JP]; 2-14-2-106, Sakae-cho, Higashimurayama-shi, Tokyo 189-0013 (JP). KIMURA, Michio [JP/JP]; 9-8-304, Tsurugadai, Chigasaki-shi, Kanagawa 253-0003 (JP). (74) Agent: VIEILLEFOSSE, Jean, Claude; Hoechst Marion Rous- sel, 102, route de Noisy, F-93235 Romainville Cedex (FR).			(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, TZ, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: <u>BONE MORPHOGENETIC PROTEIN ANTAGONIST BASED ON THE MATURE PROTEIN</u>			
(57) Abstract <p>The purpose is to provide a mature protein having an antagonistic activity against bone morphogenetic proteins. The mature protein having an antagonistic activity against bone morphogenetic proteins is obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue. The chemical modification for said methionine residue is performed by an oxidation reaction or an alkylation reaction. The chemical modification for said tryptophane residue is performed by an allylsulphenylation reaction. Or a mature protein having an antagonistic activity against bone morphogenetic proteins is obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), and mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.</p>			

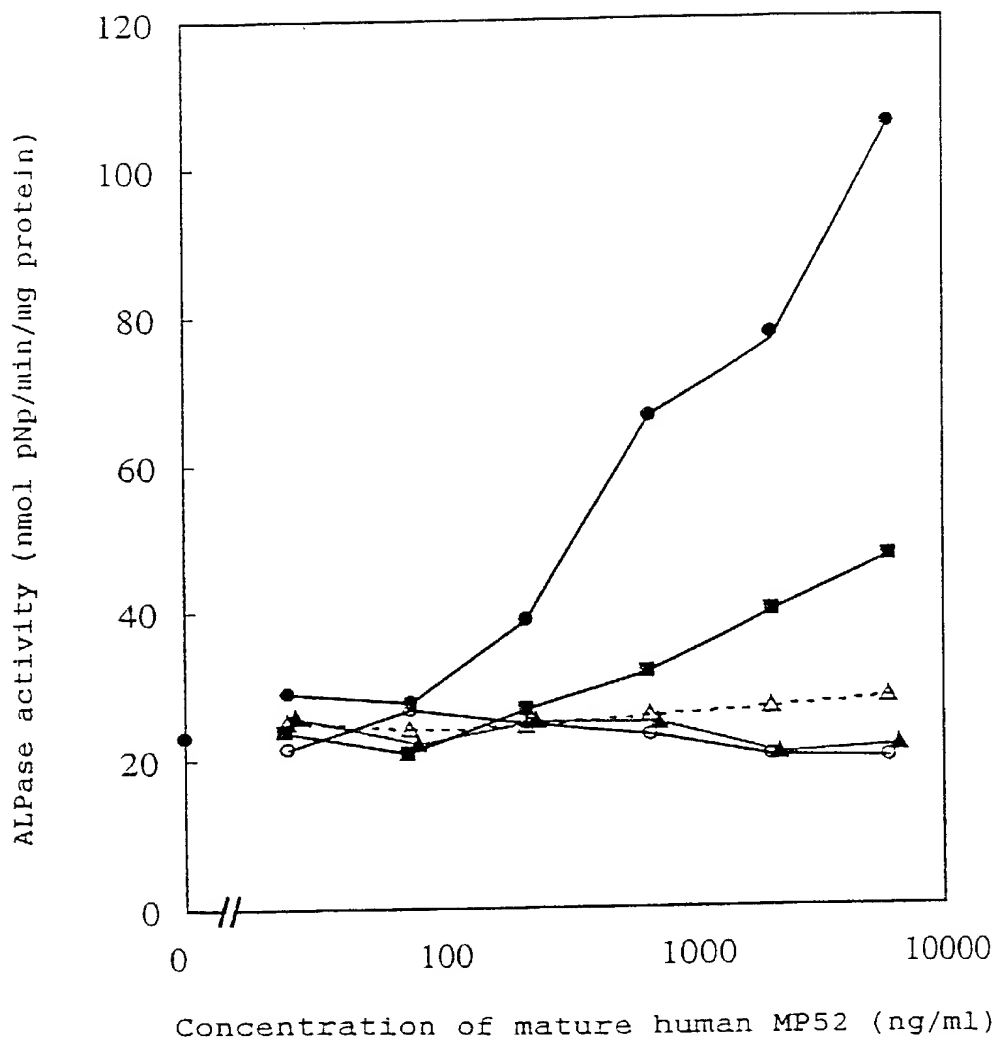


FIGURE 1

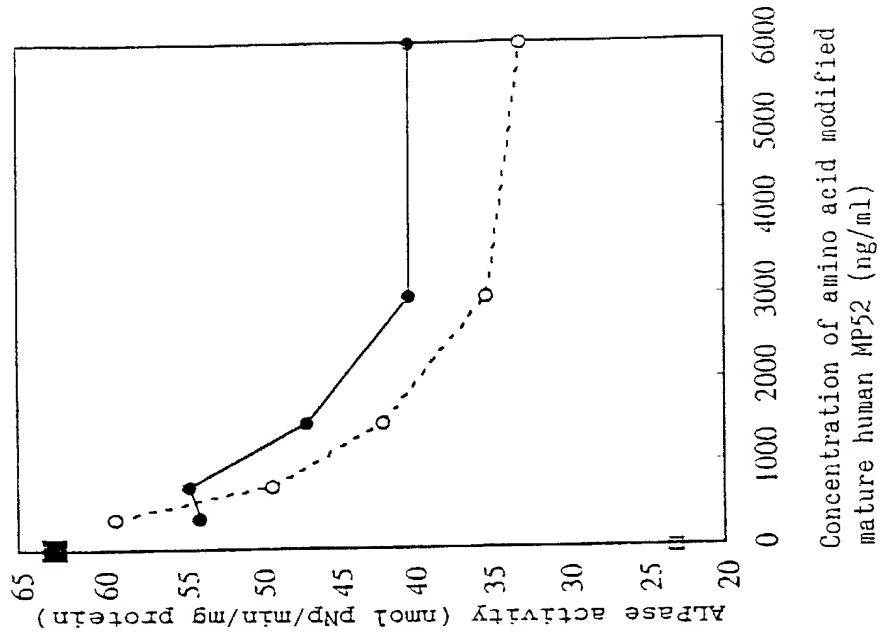


FIGURE 2B

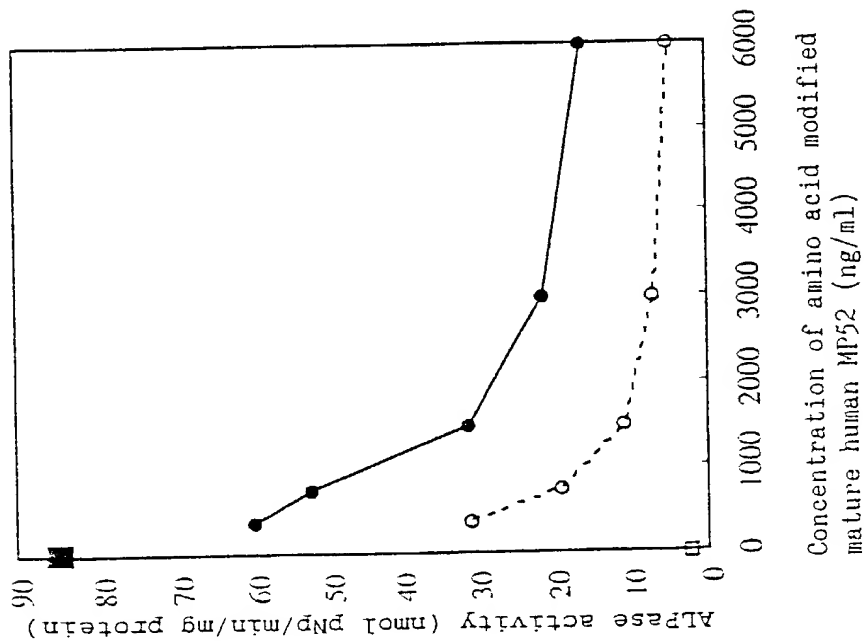
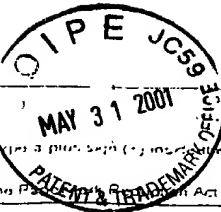


FIGURE 2A



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☒ Declaration OR
Submitted
with Initial Filing

☐ Declaration
Submitted after
Initial Filing

Attorney Docket Number	17 447.001
First Named Inventor	KATSUURA et al
COMPLETE IF KNOWN	
Application Number	PCT/IB99/01621
Filing Date	October 4, 1999
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**BONE MORPHOGENETIC PROTEIN ANTAGONIST BASED ON THE MATURE
PROTEIN**

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 10/04/99

as United States Application Number or PCT International

Application Number PCT/IB99/01621 and was amended on (MM/DD/YYYY) (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
10/288103	Japan	10/09/98	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
PCT/IB99/01621	PCT	10/04/99	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

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As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Bierman, Muserlian and Lucas	18,818		
Jordan B. Bierman	18,629		
Charles A. Muserlian	19,683		
Donald C. Lucas	31,275		

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

Direct all correspondence to:

Name	<u>Charles A. Muserlian</u>		
Address	<u>Bierman, Muserlian and Lucas</u>		
Address	<u>600 Third Avenue</u>		
City	<u>New York</u>	State	<u>NY</u>
Country	<u>U.S.A.</u>	Telephone	<u>(212) 661-8000</u>
		Fax	<u>(212) 661-8002</u>

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Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name	<u>Mieko</u>	Middle Initial		Family Name	<u>Katsuura</u>	Suffix e.g. Jr.	
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Inventor's Signature	<u>Mieko Katsuura</u>	Date	<u>April 25, 2001</u>
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Residence: City	<u>Tokyo</u>	State		Country	<u>Japan</u>	Citizenship	<u>Japan</u>
-----------------	--------------	-------	--	---------	--------------	-------------	--------------

Post Office Address	<u>2-14-2-106, Sakae-cho, Higashimurayama-shi, Tokyo 189-0013</u>		
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City	<u>Tokyo</u>	State		Zip	<u>189-0013</u>	Country	<u>Japan</u>
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Post Office Address							
Post Office Address							
City	State	Zip	Country				
Name of Additional Joint Inventor, if any:				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
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Inventor's Signature	Date						
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City	State	Zip	Country				
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Inventor's Signature	Date						
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City	State	Zip	Country				

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<301> MAKISHIMA, Fusoa
TAKAMATSU, Hiroyuki
MIKI, Hideo
KAWAI, Shinji
KIMURA, Michio
MATSUMOTO, Tomoaki
KATSUURA, Mieko
ENOMOTO, Koichi

WO 00/21998

PCT/IB99/01621

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<301> WANG, Elizabeth A.

WOZNEY, John M.

ROSEN, Vicki A.

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<312> 1988-01-14

<313> 1 TO 114

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Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln

50

55

60

Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val

65

70

75

80

Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu

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105

110

Cys Arg

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WO 00/21998

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35 40 45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His

50 55 60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro

65 70 75 80

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe

85 90 95

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val

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